REGENERATING TISSUES FROM THE COCKROACH, LEUCOPHAEA MADERAE: NERVE REGENERATION IN VITRO [1]

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In an earlier study, Marks and Reinecke (1965) reported the migration of cells from the nerve stump of isolated leg regenerates of the Madeira cockroach, *Leucophaea maderae* (F.), and demonstrated that this growth did not follow the cyclical pattern associated with the molting process that was characteristic of epithelial tissues from the regenerating leg. In the same study, an isolated incident was observed and recorded on film in which cell processes grew out from the proximal end of the 5th mesothoracic nerve of an isolated leg regenerate and made contact with a ganglion that had been placed adjacent to it. Wigglesworth (1965) stated that during post-embryonic development, sensory axons *in vivo* normally differentiated and migrated inward to invade the central ganglion while motor axons emerged from the ganglion itself, and Bodenstein (1957) suggested that the same processes occurred during the healing of a severed nerve. Guthrie (1962, 1967) and Jacklet and Cohen (1967) demonstrated that regeneration of transplanted ganglia and the healing of mesothoracic nerves takes place *in vivo* in the adult cockroach. The recent development of a nutrient medium—M-18 (available from Grand Island Biological Co., Grand Island, N. Y. 14072)—that favors nerve growth and development therefore encouraged us to repeat our earlier *in vitro* experiments to study nerve regeneration in detail. In the present study, we attempted to correlate the regenerative growth processes as observed *in vitro* with normal nerve regeneration as found *in vivo* by other workers.

Materials and Methods

The technique for preparing leg regenerates was described in detail by Marks (1968). The mesothoracic legs of late instar nymphs were cut off at the trochantero-femoral articulation 24 hours after molting. Regeneration was allowed to proceed for 8 days, at which time the entire coxa was removed from the insect, and the leg regenerate was dissected under sterile conditions; at the same time, the prothoracic ganglion and gland were removed. The explants, two leg regenerates and a single ganglion, were washed in M-18 nutrient medium, placed in a Rose multipurpose tissue chamber under a strip of dialysis membrane, and the explants so oriented that the stump of the 5th mesothoracic nerve projecting proximally from the leg regenerate was adjacent to the stumps of the lateral nerves

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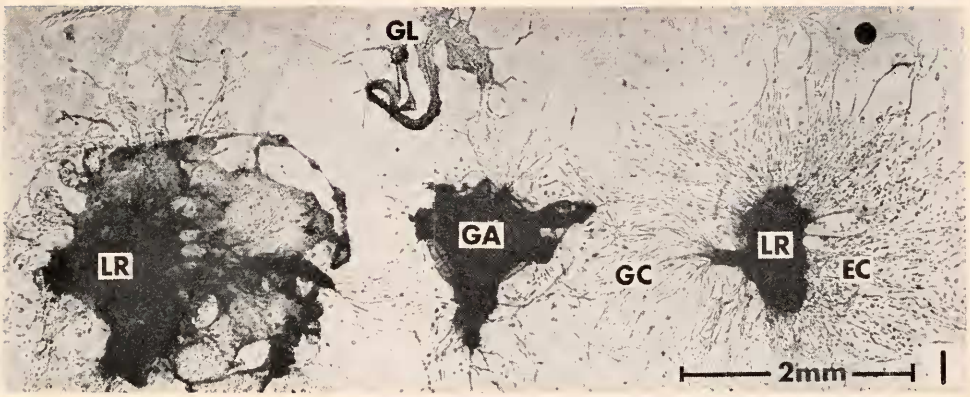

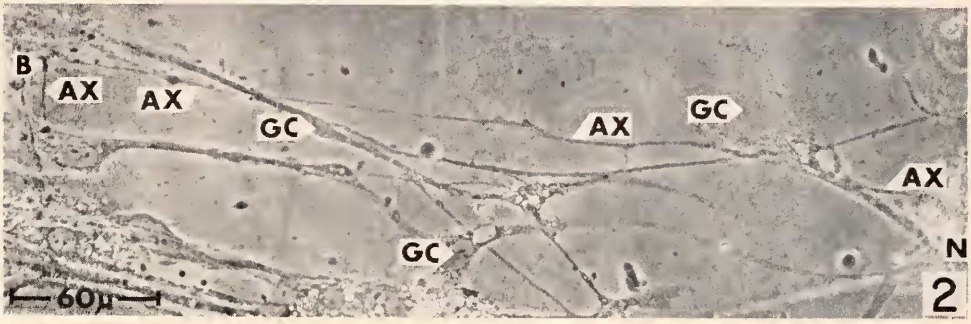

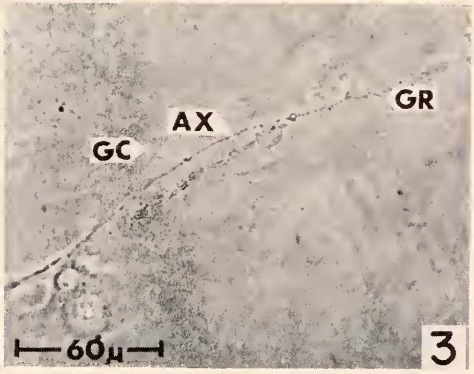

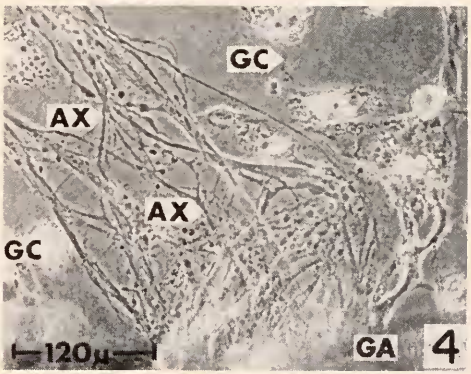

FIGURE 1. Culture containing leg regenerates (lr), prothoracic ganglion (ga), and prothoracic gland (gl). The leg regenerates are surrounded by partial sheets of epithelial cells (ec). Glial cells (gc) are seen emerging from the cut ends of the nerve of both leg regenerates and ganglion and forming a cell bridge. *In vitro* 21 days; transmitted light.

FIGURE 2. Typical growth from the nerve end in a leg regenerate. Sensory axons (ax) emerge from the severed nerve (n) and maintain close contact with several glial cells (gc). *In vitro* 37 days; dark contrast phase.

FIGURE 3. Schwann-like glial cell (gc) with sensory axon (ax) running along the surface. Time-lapse studies show much activity in the region of the growth cone (gr). *In vitro* 60 days; dark contrast phase.

FIGURE 4. Axon (ax) fibers emerging from ganglion explant (ga) migrate through a break in the neurilemma. Glial cells (gc) containing lipid-filled vacuoles are also emerging. *In vitro* 26 days; dark contrast phase.

of the ganglion (Fig. 1). The distance between these tissues was not allowed to exceed 1 mm. In some cases, the prothoracic gland was placed adjacent to the ganglion. The chamber was set up with one glass and one plastic coverslip, and the explants were held against the glass coverslip by the dialysis strip. The completed chamber was filled with M-18 that contained 7.5% fetal calf serum. Another series was prepared with chambers containing only ganglia or leg regenerates. The explants were examined weekly under a phase contrast microscope with a long working distance condenser. If axons and glial cells emerged from the explant, it was scored as positive; if not, it was scored as negative. Only explants that could be clearly scored were included. The results of each experiment were tested against the others by using a test for difference between two sample proportions given by Goldstein (1964). Significance was set at the 95% level of confidence.

To fix and stain these cultures for identification of the cell types, we found it necessary to disassemble the Rose chamber and remove the glass coverslip with its associated dialysis strip. Since the cultures grew between the coverslip and the dialysis strip, extreme care was taken to preserve the delicate structures. First, the 45 × 50-mm. coverslip was cut to a 1-in. width with a diamond pen and then the coverslip was attached, together with the undisturbed dialysis strip, to a slide with metal clips. The entire assembly was then fixed in Gendre's fixative for two hours, stained by the periodic acid-Schiff technique, followed by 5 minutes in Hansen's trioxyhematin. Additional explants were pretreated with either alpha-amylase or pepsin before they were stained by the PAS method. After dehydration and clearing, the dialysis strip was so heavily stained that it was necessary to remove it. In most cases, the explant and associated cell outgrowth adhered to the coverslip though occasionally the explant adhered to the dialysis strip and was pulled away with it. When this occurred, the cell outgrowth remained on the coverslip and could be studied after mounting. The trioxyhematin provided a delicate stain that outlined both the nucleus and cytoplasm, so the location of the PAS-positive granules in the cell was easily determined.

Results

The first activity seen in the explanted leg regenerates occurred within 24 hours when the blood cells migrated onto the coverslip; they frequently formed an imperfect monolayer around the leg regenerate. Within 10 days, epithelial cells were migrating from the explant, and a few days later, large granular cells with long attenuated processes migrated from the nerve sheath or neurilemma and often became very long and spindle-shaped. These probably represent glial cells. They could be distinguished from neurons by their granular cytoplasm and by migration of their cell bodies from the explant across the glass. They often joined in long strings that moved over the coverslip and eventually made contact with cells from other explants, either blood cells or other glial cells (Fig. 1). Examination of the cell surfaces frequently revealed fine fibers of darker contrast. When we traced these fibers, we found that they extended from the interior of the nerve stump. The fibers were morphologically distinct from the glial cells and possessed growth cones identified as sensory on the basis of

work done by Pomerat (1967) and Reinecke (personal communication); the apparent location of the cell body in the leg regenerate (*i.e.*, distal to the cut) further identified them as sensory fibers. They sometimes made contact with blood cells as they moved from the explant, but they were usually found closely associated with the glial cells (Fig. 3). In a few cases, they crossed the cell bridge between explants.

The first activity seen in the explanted prothoracic ganglia appeared 12 to 14 days after explantation: blood cells were usually present, but few epithelial cells and no cell sheets were formed around the ganglion. By the 14th day, two types of fibers were migrating from the explant. One emerged from the body of the ganglion through breaks in the neurilemma, and they migrated freely over the glass surface and formed extensive networks. Both blood and glial cells were contacted by these fibers, the growth cones of which were similar to those identified as motor types by Pomerat and Reinecke. The cell bodies of these fibers always remained within the ganglion. Thick ropelike strands often formed and bridged the gap between explants and invaded the tissues of the leg regenerates (Fig. 4).

A second type of fiber migrated from the cut ends of the lateral nerves of the ganglion in close association with the glial cells. They were morphologically indistinguishable from the fibers described earlier from the leg regenerate and were assumed to be axons from association neurons within the ganglion. The location of the cell bodies of these fibers is not known.

In the 14 growth chambers containing one ganglion and two leg regenerates, 81% of the 23 leg regenerates that could be scored and 85% of the 14 ganglia showed fiber growth. Cell bridges between the ganglion and one or both the leg regenerates developed in 64% of the 14 chambers (Fig. 5).

Maximum development of the cultures occurred at 25 to 30 days *in vitro;* after this, the supporting cells began to degenerate. The larger groups of nerve fibers persisted longer and remained as a network that could be identified when freed from the accompanying blood and glial cells (Fig. 6). Axoplasmic traffic was clearly visible in time-lapse photomicrographic studies of these fiber bundles. The makeup of the bundles in older chambers was difficult to ascertain in the living material; sensory, motor, or both types of fibers may be involved. A few glial cells usually remained though no well-defined sheath was observed, and there was no evidence of the secretion of a neurilemma. In some of the chambers, such connecting fibers had formed into compact, cable-like structures that resembled a nerve trunk; in others, they remained diffuse. Such nerve-mimicking structures with recognizable axons and glial cells were found as long as 80 days after explantation.

When a prothoracic gland was present with the ganglion, nerve fibers from the ganglion frequently made contact with the gland. The prothoracic gland remained alive and put out cell migrants, and its presence had no visible effect on the activity of either glial cells or nerve fibers.

Of the leg regenerates set up with the ganglion and prothoracic gland, 87% of 16 produced nerve fibers and support cell migrants; of those set up without the gland, 81% of 23 showed such growth. The ganglion explants had a similar pattern: 86% of 13 showed cell migration in the presence of the gland and 85%

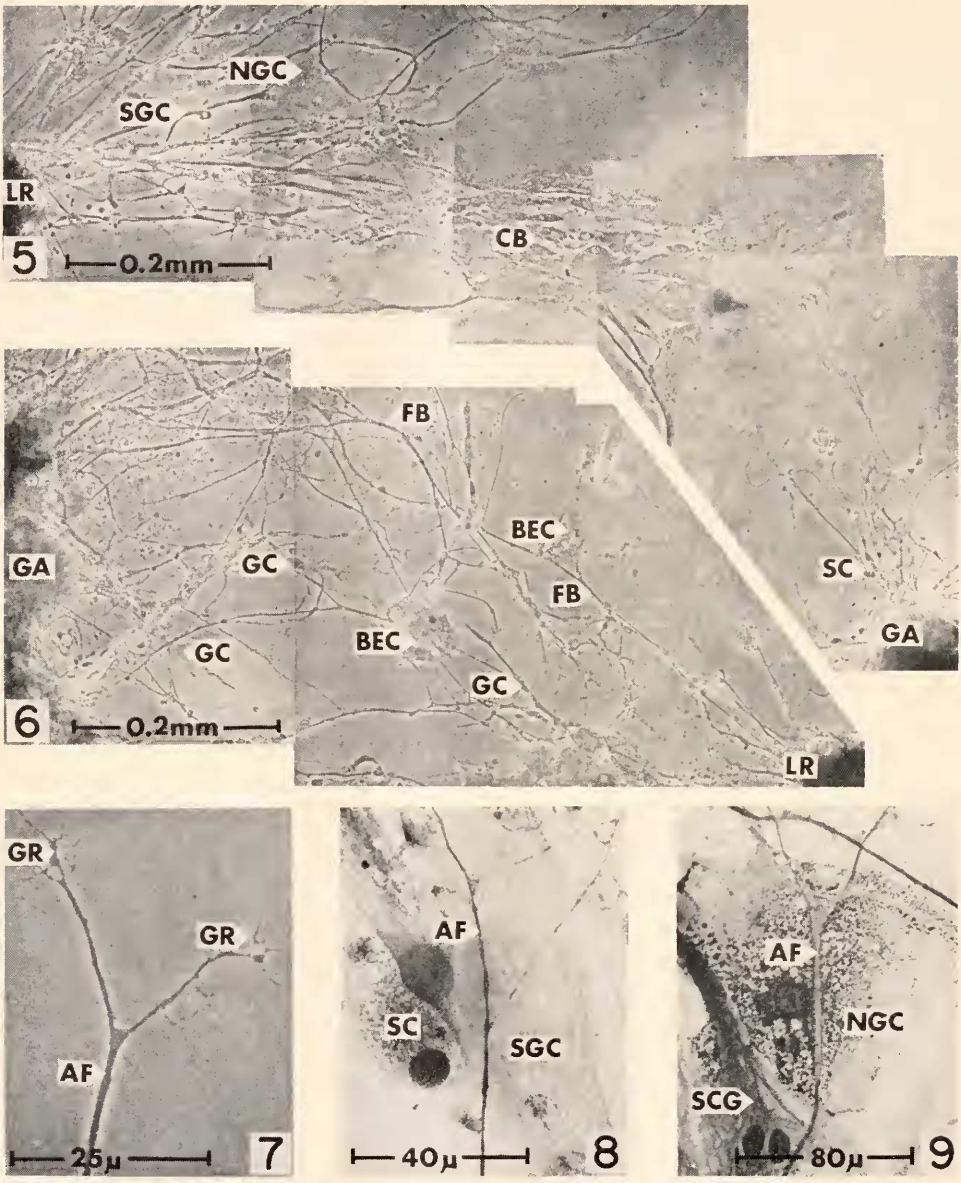

FIGURE 5. Cell bridge between leg regenerate (lr) and ganglion explant (g) is developing. Elongated Schwann-like glial cells (sgc) and granule-filled nutritive type glial cells (ngc) are emerging from the explants. What may be a sheath cell (sc) is seen near the ganglion explant. The center portion of the bridge (cb) appears to be made up of a mixture of blood, glial, and epithelial cells. *In vitro* 34 days; dark contrast phase.

FIGURE 6. Degeneration of blood and epithelial cells (bec) in old cultures exposes the mature nerve net that has formed. A few recognizable glial cells (gc) remain associated with the fiber bundles (fb). *In vitro* 62 days; dark contrast phase.

of 14 in its absence. A summary of these results is given in Table I. Thus, in this test, the presence of the gland had no apparent effect on the occurrence of nerve regeneration.

To check these observations, we made a second series of experiments in which homologous explants from different animals were placed together in a single chamber. Both leg regenerates (four to a chamber) and prothoracic ganglia (three to a chamber) were prepared as described. The chambers contained about the same amount of tissue as those described earlier. Both kinds of tissue grew quite readily. In chambers containing only leg regenerates, the glial cells and fibers migrated in 44% of the 23 explants. Cell bridges of blood and glial cells that carried axons were formed, but they did not reach and penetrate into other explants, and no lasting fiber aggregates were formed.

TABLE I

The effect of the presence of various tissue combinations on nerve regeneration in vitro

Tissue combinations	Test organs:			
	Leg regenerate		Prothoracic ganglion	
	% growth	Total scored	% growth	Total scored
Leg-ganglion-leg	81	23	85	14
Leg-ganglion+gland-leg	87	16	86	13
4 Leg regenerates	44	23	—	—
3 Ganglia	—	—	90	20

The chambers provided an excellent opportunity to study the regeneration of the sensory type fibers since no motor cell bodies were present in the isolated leg regenerate. The long, slender axons were phase dark and could be seen against the lighter cytoplasm of the glial cells. A single axon sometimes varied throughout its length, broadening and then abruptly narrowing again. Irregular branching occurred, and slender filaments frequently extended laterally at irregular intervals. With time-lapse cinephotomicrography, the growth cone portion of the axon was extremely active, and at times, it extended outward from the advancing glial cell. As the growth cone proceeded along the surface of a sheath cell, it formed flattened droplets of cytoplasm that appeared to provide connections

FIGURE 7. Branched axon (af) fiber with two growth cones (gr) that are probably sensory in origin but have lost some of their identifying characteristics. This fiber branched from the single large axon bundle that joined a leg regenerate to the ganglion. *In vitro* 74 days; dark contrast phase.

FIGURE 8. Stained preparation showing: a sheath cell (sc), a Schwann-like glial cell (sgc) with cytoplasmic fibers, typical nuclear morphology, and associated axonal fiber (af).

FIGURE 9. Stained preparation showing two Schwann-like glial cells (sgc) adjacent to a nutritive glial cell (ngc) filled with PAS-positive granules. Nuclear morphology of these two cell types is virtually identical. Axonal fibers (af) are closely associated. *In vitro* 50 days.

with the membranes of the sheath cells. Mitochondria and refractile granules were occasionally seen in the axoplasm.

In chambers containing only ganglia, migration of nerve fibers occurred in 90% of the 20 explants, and both types of nerve fibers were present. The results of these experiments are summarized in Table I. In such chambers, particularly those 40 or more days *in vitro*, fibers presumably from association neurons often migrated independently; however, when a glial cell was encountered, the fiber frequently followed it for a considerable distance. In the older chambers, trunk-like aggregates of fibers were frequently formed, possibly as a consequence of the thigmotropic behavior of these cells.

In preparations of young ganglia (15 to 30 days *in vitro*), fibers that were probably motor fibers, as judged by the structure of their growth cones and by the fact that structures of this type were absent in leg regenerate preparations, were found growing mostly from the body of the ganglion through breaks in the neurilemma. They grew out onto the glass surface where the individual fibers formed angular, jointed patterns and loops, and later, as they matured, fiber bundles. In the older chambers (30 to 45 days), it became more difficult to identify the fibers, and growth cones of intermediate types were frequently found (Fig. 7). Cell bridges formed between individual ganglion explants within the same chamber.

While the phase contrast microscope allowed us to ascertain that the cells involved in forming cell bridges were of more than one type, we could not always distinguish clearly between the different types of cells. Several ganglion cultures were therefore fixed and stained for study.

The nerve fibers and glial cells stained readily with Hansen's trioxyhematin, and the neurilemma of the ganglion yielded a positive reaction when it was stained by the PAS technique. The sheath appeared to be lined with small cells containing densely staining nuclei with the chromatin scattered in coarse granules. These cells, often binucleate, occasionally migrated onto the coverslip where they could be identified by their small, dark-staining nuclei (Fig. 8). The cytoplasm was densely packed with PAS-positive granules that were unchanged by digestion with either alpha-amylase or pepsin. The cells occurred in relatively small numbers and fitted Wigglesworth's (1959) description of type I glial cells that belong to the perineurium.

A second type of migrating cell appeared in greater numbers, were much larger, and contained a large, lightly granulated nucleus with one or two prominent nucleoli. The cytoplasm was hyaline around the periphery and frequently included large numbers of granules in the perinuclear region that were also PAS-positive after digestion with alpha-amylase and pepsin (Fig. 9). These cells had an alveolar texture to their cytoplasm, frequently with areas along the periphery that appeared to contain numerous small vacuoles that were also PAS-positive. These cells are one of the components of the cell bridges that form between explants.

A third type of cell present in large numbers varied greatly in shape and was characterized by the presence of numerous fibrils in the cytoplasm and by the absence of PAS-positive granules. They were very large, and some developed long fibrous processes. They were usually the first cells to migrate from a fresh

explant, and they were the main components of the cell bridges (Fig. 8). They were also the cells with which the growth cones of the nerve fibers were most commonly associated as they migrated from the explant. Both the second and third types of cells appeared to belong to the type II category of glial cells (Wigglesworth, 1959) and appeared to be actively involved in the process of nerve regeneration. The second type with its PAS-positive granules may play a nutritive role (Wigglesworth, 1960); the third type with its long, fibrous processes and intimate association with the nerve fibers may play the role of Schwann-like cells. It is likely that these two types have a common origin, the morphological differences being dependent on the physiological role of a particular cell at given time since intermediate types are occasionally found.

Discussion

The nerve cell bridges formed in our *in vitro* studies closely resembled those described by Bodenstein (1957). So well, in fact, does his description of *in vivo* regeneration fit the activities found in the present studies that there can be little question that the *in vitro* findings are representative of the process of regeneration as it normally occurs. In both cases, (a) fibers emerge from the proximal and distal nerve stumps and cross a cell bridge made up of blood and connective tissue cells, (b) fiber bundles can be traced from the proximal to the distal nerve stump, (c) many fibers become lost wandering from the target tissues and sometimes form loops and coils that go nowhere, (d) fibers from ganglia and leg regenerate stumps often reunite with other ganglia or leg stumps instead of forming a union between the distal and proximal stumps, and (e) fibers from the distal and proximal stumps may meet in the middle of the cell bridge and form a reticulum of fibers.

Bodenstein also pointed out that nerve regeneration occurred in adult insects and was independent of the molting cycle and thus of the influence of the prothoracic gland. In our study, the prothoracic gland had no visible effect on the growth of nerve fibers from either the leg regenerate or the prothoracic ganglion. This evidence strongly supports the earlier conclusion of Marks and Reinecke (1963) that nerve regeneration is a noncyclical or wound-healing type of growth rather than the cyclical, molting-dependent type found in nymphal epithelial tissues.

One question was whether observable interactions occurred between the leg regenerate and the ganglion tissues. In the cultures containing both types of tissues, fiber growth occurred in 81% of the leg regenerates and 85% of the ganglia. When these same tissues were cultured in isolation, 44% of the regenerates and 90% of the ganglia showed such growth. The results are similar to those from an earlier study (Marks and Reinecke, 1965) in which fiber growth was found in 64% of 11 regenerates when the ganglion was present and in 29% of 24 regenerates when the ganglion was absent. While neither tissue required the presence of the other in order to initiate regenerative growth, the presence of the ganglion significantly increased the occurrence of nerve growth in leg regenerates. However, the presence of the leg regenerate had no measurable effect on the growth from either the ganglion or the nerve stump of other leg regenerates

in the chamber. This is evident when these results are compared with those of the 1965 studies. In the earlier study, only two leg regenerates were present in the control chambers, while in the present study, there were four. Yet, in both studies, the nerve growth in the chambers containing only leg regenerates was approximately 50% of that in chambers containing both leg regenerates and ganglia.

Another question concerned the directional orientation of the fibers as they emerged from the explants. During the first few days, the sensory fibers emerging from the distal nerve end in the leg regenerates grew parallel to the shaft of the nerve stump so that they appeared to have a directional orientation if the adjacent explants were close together; however this orientation subsided with time, and additional fibers emerged and grew in numerous directions, apparently independent of any directional influences. Both sensory and motor fibers appeared to have a tactile or thigmotropic response that caused them to follow the cell bridges formed by the blood and sheath cells. If the explants were over a millimeter apart or if no cell bridge formed, most fibers became lost and migrated in a random manner over the glass surface.

One additional phenomenon noted by Bodenstein (1957) was confirmed: those fibers that did eventually make contact with another explant appeared to grow in diameter and persisted in old cultures after the surrounding tissues had broken down. This can be partially explained by the thigmotropic response of the fibers causing bundles to form as new fibers migrated along the surface of the old ones. The migration of fibers from both explants over the same bridge would account for the greater size when compared with bundles formed when fibers migrated from one source only and reached a dead end.

Conclusions

1. The regeneration of nerve tissues which occurs *in vitro* is comparable, within limits, to that which occurs *in vivo*. Both sensory and motor nerve elements take part in the regenerative process. Blood and glial cells play an important role in forming the cell bridge that facilitates the migration of the nerve axons.

2. The presence of ganglion tissue in the growth chamber exerts a demonstrable stimulating influence on the outgrowth of cells from the nerve stump of the leg regenerate. However, the presence of the leg regenerate appears to have no effect on the outgrowth of cells from the ganglion.

3. A tactile or thigmotropic type of behavior by the migrating axons is evident that explains several of the phenomena described.

4. With such similarity between the development *in vivo* and *in vitro,* the advantages of the *in vitro* technique become apparent. High-resolution photography of the activities of single cells is possible, and the activity can be followed for many days by using time-lapse cinephotomicrography. Also, great experimental flexibility is possible because there need be no interference from other tissues. In addition, tissues from several insects may be included in a single chamber which may make it possible to investigate a number of phenomena that are not otherwise accessible for experimentation.

LITERATURE CITED

Bodenstein, D., 1957. Studies on nerve regeneration in *Periplaneta americana*. *J. Exp. Zool.*, **136**: 89–116.

Goldstein, A., 1964. Biostatistics. First Ed., p. 101, Macmillan Co., N. Y.

Guthrie, D. M., 1962. Regenerative growth in insect nerve axons. *J. Insect Physiol.*, **8**: 79–92.

Guthrie, D. M., 1967. Regeneration of motor axons in an insect. *J. Insect Physiol.*, **13**: 1593–1611.

Jacklet, J. W., and M. J. Cohen, 1967. Synaptic connection between a transplanted insect ganglion and muscles of the host. *Science*, **156**: 1638–1640.

Marks, E. P., and J. P. Reinecke, 1965. Regenerating tissues from the cockroach, *Leucophaca maderae:* Effects of endocrine glands *in vitro*. *Gen. Comp. Endocrinol.*, **5**: 241–247.

Marks, E. P., 1968. Regenerating tissues from the cockroach, *Leucophaca maderae:* The effect of humoral stimulation *in vitro*. *Gen. Comp. Endocrinol.* (in press).

Pomerat, C. M., W. J. Hendelman, C. W. Raiborn, Jr., and J. F. Massey, 1967. Dynamic activities of nervous tissue *in vitro*. *In:* The Neuron, Chapt. III, pp. 119–178. H. Heyden ed., Amer. Elsevier Publ. Co., N. Y.

Rose, G., 1954. A separable and multipurpose tissue culture chamber. *Tex. Rpt. Biol. Med.*, **12**: 1074–1083.

Wigglesworth, V. B., 1959. Histology of the nervous system of an insect, *Rhodnius prolixus* (Hemiptera). *Quart. J. Microscop. Sci.*, **100**: 299–313.

Wigglesworth, V. B., 1960. Nutrition of the central nervous system in the cockroach, *Periplaneta americana* L. *J. Exp. Biol.*, **37**: 500–512.

Wigglesworth, V. B., 1965. Principles of Insect Physiology, 6th Ed. Methuen and Co., London.